Brood Pheromone Can Modulate the Feeding Behavior of *Apis mellifera* Workers (Hymenoptera: Apidae)

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ABSTRACT A honeybee brood pheromone, made of 10 simple fatty aliphatic esters previously described as important compounds in the chemical communication between brood and workers, was tested as an additional chemical stimulus in the artificial rearing of the queens. Three of these compounds significantly affected queen rearing when they were applied in amounts similar to those naturally found on larval cuticle. Methyl stearate increased the acceptance of the queen cells, methyl linoleate enhanced the amounts of royal jelly deposited by the worker, and methyl palmitate improved the weight of the larvae. The role of these compounds in the chemical ecology of the larval feeding process and their practical use in beekeeping is discussed.

KEYS WORDS *Apis mellifera*, brood pheromone, feeding behavior

IN *Apis mellifera* L. colonies, adults take care of the immatures (eggs, larvae, and pupae) and provide food to the larval stages. The feeding behavior of the worker honey bees, known as nurses, varies on a qualitative and a quantitative level as a function of age and caste of the larvae (reviewed by Haydak 1961, Lercker et al. 1984, Brouwers et al. 1987). Qualitative modulation of the jelly given by the nurse determine whether a 3-d-old (or younger) female diploid larva will become a worker or a queen (Jung-Hoffmann 1967; Brouwers 1984; Asencot and Lensky 1985, 1988). The modulations of feeding behavior suggest that the nurses could distinguish between young and old larvae, as well as between the different larval castes. Different cues, such as mechanical or chemical stimulus, or both, can be involved in that discrimination, but still are not known.

Mechanical factors, such as the feature of the cell, are implied in the recognition of three types of cells (worker, drone, and queen) and are involved in the feeding behavior of the nurses (Gontarsky 1949, review by Pain 1961). Free and Winder (1983) showed that physical characteristics of the brood are of relatively little significance, and gave evidence of the existence of a contact brood pheromone that could be the prime brood recognition signal. The larvae emit a general signal indicating their presence in the cell to the worker bees, which can also recognize their state of starvation, probably using another stimulus intrinsic to the larva (Free et al. 1989; Huang and Otis 1991a, b). Other cues, related to the quantity of food in the cell, could also modulate food deposition (Huang and Otis 1991b).

The nature of chemical signals involved in the feeding of the larvae by the worker bees is still unknown. Recently, 10 simple aliphatic esters were identified on drone larvae (Le Conte et al. 1989). Four of them, methyl palmitate, methyl linoleate, methyl linolenate, and methyl oleate are components of a brood pheromone, which induce the workers to cap the cells containing mature larvae (Le Conte et al. 1990). Methyl linoleate, methyl linolenate, and methyl oleate are present on the cuticle of the queen pupae and are involved in the recognition of the queen cells by the workers (Le Conte et al. 1995). The 10 compounds, also identified on young and old queen larvae as well as on worker larvae, are emitted in large amounts during the capping of the cell and in different amounts and relative proportions depending on the age and the caste of the larvae (Trouiller et al. 1991, Trouiller 1993). Modulation of the proportions and quantities of these 10 esters, as a function of the larval age, constitutes a chemical signature that enables adult workers to recognize young or old larvae (Le Conte et al. 1994). Because the fatty acid esters are involved in brood recognition by the bees, we have investigated their role in the modulation of the feeding behavior of the worker bees and used these compounds as additional chemical stimuli in the rearing of queens.

The purpose of our experiments was to determine if including the fatty acid esters in the wax of the cups used in queen rearing would affect the number of larvae accepted, the amount of royal jelly put into the cup by the workers, as well as the weight of the larvae produced in the different cups. First, we screened the efficiency of different

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esters, then we developed dose–response experiments for each active ester.

Materials and Methods

Hybrid colonies of *A. mellifera* (*ligustica* × *caucasica*) × *mellifera* were used between May and July to rear queens, using standard commercial methods (Laidlaw and Eckert 1962, Fresnaye 1975). Beehives were separated into two parts with a queen excluder to keep the queen in one part, while workers could go in the two different parts through the apparatus.

Queens were reared and produced royal jelly using artificial queen cells made of wax cups. A drop (≈20 μl) of diluted royal jelly was placed in the bottom of a cup, and a 1- to 3-d-old worker larva was transferred to the cup, and floated on the royal jelly (grafting). Approximately 40–50 such cups are placed in a queenless part of a colony to be reared by workers. Three days after grafting the larvae, the queen cells are withdrawn from the colony for the harvest of royal jelly.

Wood bars (42 by 2 by 0.5 cm), each containing 25 wax cups, were used. Two bars were placed in a frame to be introduced in the queenless part of the colony, and the 50 wax cups each containing a grafted 1- to 2-d-old worker larva were introduced to the workers for queen rearing.

One of the 10 fatty acid esters, or the reconstituted blend corresponding to one characterized by Trouiller et al. (1991) for 8.5-d-old worker larvae, was included in the wax of each cup as follows: esters were mixed with wax at 50°C and the top of a stick of wood, shaped like the interior part of a queen cell, was immersed in the mixture for a few seconds. Once cold, the wax cup was withdrawn from the wood and sealed on the bar with hot honey bee wax.

In the first experiment, a screening of the effects of the different esters was performed at three concentrations: 10^{-2}, 10^{-3}, and 10^{-4} of ester in wax (wt:wt), corresponding to 10^3, 10^2, and 10 μg of ester per cup. One of each different wax cup, with different esters and concentrations, was tested at the same time in the same colony. Wax cups without esters were tested as controls. Eight colonies were used. The experiment was repeated at least 55 times per ester at each concentration.

After 3 d, workers had naturally destroyed a variable proportion of larvae and their wax cup. We recorded the number and the type of wax cups accepted by the workers, and we weighed the royal jelly and the larvae contained in each cell.

To confirm the results of the first experiment, we performed a second experiment using three esters: methyl palmitate, methyl stearate, and methyl linoleate at the concentration of 10^{-3} (wt:wt). Twelve control cups were placed on one-half of the bars and compared with 12 other cups, all containing the same ester, on the other half of the bar (to limit the attraction effect of the cups including esters, we did not alternate control and treated cups). Two bars were tested for each colony. The experiment was repeated in different colonies, at least five times per ester tested. For each combination, at least 80 accepted cups with one of the four esters were compared with at least 80 accepted control cups.

In a third experiment, dose–response tests were performed separately with methyl linoleate, methyl palmitate, and methyl stearate. For each ester, wax cups at the concentration of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} (wt:wt) and 0 (control) were placed at random on two bars and tested in a colony. One ester at a time was tested with different concentrations in a colony; at least eight replicates were done per ester.

Statistical tests were performed using the SAS Institute (1992) statistical package. Tests employed included chi-square for the acceptance of the wax cups and analysis of variance general linear model (GLM) with mean separations by Fisher's least significant difference test (not protected LSD) for the weight of royal jelly and the weight of the larvae.

Results

In the first experiment, there was no colony effect in the acceptance of the wax cups, which varied from 48 to 53% among the different colonies. The acceptance of the wax cups by the workers varied from 46.1 to 79.2%, depending on the compound of the cup. Three of the 33 combinations, methyl stearate 10^{-3} (wt:wt), methyl oleate 10^{-4} (wt:wt), and methyl palmitate 10^{-3} (wt:wt) led to significantly higher acceptance in comparison to the control cups (Table 1). Compared with the control cups, worker bees accepted more cups with methyl stearate, when we consider the three concentrations together (Table 2).

Different amounts of royal jelly were produced per accepted cup in the different rearing colonies ($F = 75.25$, $P < 0.0001$). The effect of the type and of the ester concentration was not significant on the amounts of royal jelly, respectively ($F = 1.17$, df = 11, $P < 0.29$) and ($F = 0.92$, df = 2, $P < 0.40$), but the position of the bar had an effect ($F = 72.9$, $P < 0.0001$). On the bars, per accepted cups, 298.4 ± 4.7 mg (mean ± SEM) of royal jelly were produced at the top of the frame, and 329.9 ± 3.8 mg at the bottom of the frame. When the amount of royal jelly was more important per accepted cup with methyl linoleate 10^{-4}, in that case, significantly less larvae were accepted, and the larvae produced were not heavier than control larvae (Table 1). Methyl linoleate cups contained more royal jelly compared with other esters or control cups (Table 2).

The weight of larvae varied distinctly in the different colonies ($F = 13.41$, $P < 0.0001$). There was an ester effect ($F = 1.79$, $P < 0.04$), and methyl palmitate gave heavier larvae than controls (Table 2), but there was no significant effect of the ester

Table 1. Effects of the esters included in the wax of artificial queen cells at three different concentrations on the rearing behavior of the workers

Ester	Ester concn (wt:wt)	Worker acceptance of cells (%)	Royal jelly/ accepted cell (mg) (mean ± SEM)	Wt of larvae (mg) (mean ± SEM)
Control		63.3	306.6 ± 19.9	61.1 ± 6.6
	1/100	54.7	306.7 ± 19.7	60.7 ± 6.9
10 Esters	1/1,000	70.9	291.9 ± 24.3	64.9 ± 9.7
	1/10,000	68.5	300.9 ± 20.9	61.6 ± 9.0
	1/100	65.3	332.9 ± 22.5	73.3 ± 8.5
Ethyl linoleate	1/1,000	59.2	290.0 ± 23.0	58.4 ± 9.2
	1/10,000	59.6	316.7 ± 27.8	59.4 ± 8.5
	1/100	70.9	314.2 ± 25.7	68.2 ± 8.1
Ethyl linolenate	1/1,000	67.3	322.3 ± 23.8	75.9 ± 9.2
	1/10,000	69.0	321.5 ± 22.4	52.0 ± 6.6
	1/100	60.0	317.7 ± 24.1	56.8 ± 7.9
Ethyl oleate	1/1,000	71.1	307.5 ± 20.0	54.1 ± 8.4
	1/10,000	73.0	321.8 ± 23.5	63.1 ± 7.5
	1/100	53.8	303.4 ± 22.7	55.2 ± 7.5
Ethyl palmitate	1/1,000	67.3	320.4 ± 22.3	51.8 ± 6.0
	1/10,000	65.4	301.4 ± 22.5	64.6 ± 7.8
	1/100	63.4	303.1 ± 22.0	55.5 ± 7.2
Ethyl stearate	1/1,000	65.4	303.4 ± 21.6	59.9 ± 8.8
	1/10,000	63.6	334.5 ± 17.6	65.3 ± 6.8
	1/100	63.4	332.2 ± 23.3	59.2 ± 7.9
Methyl linoleate	1/1,000	66.6	335.8 ± 21.9	64.0 ± 7.9
	1/10,000	46.1*	343.9 ± 22.4*	64.4 ± 7.0
	1/100	55.7	318.5 ± 24.1	72.7 ± 9.8
Methyl linolenate	1/1,000	68.6	294.1 ± 22.0	69.8 ± 9.2
	1/10,000	57.6	311.2 ± 19.1	59.6 ± 7.6
	1/100	67.2	330.1 ± 20.4	56.9 ± 7.9
Methyl oleate	1/1,000	56.6	298.9 ± 27.5	56.1 ± 7.1
	1/10,000	78.8*	320.1 ± 19.4	57.4 ± 7.0
	1/100	61.5	309.7 ± 24.7	92.3 ± 12.8*
Methyl palmitate	1/1,000	78.8*	310.0 ± 21.9	69.7 ± 8.6
	1/10,000	69.2	318.1 ± 18.6	70.5 ± 7.3
	1/100	75.4	307.9 ± 22.6	59.6 ± 7.9
Methyl stearate	1/1,000	79.2*	312.9 ± 27.0	53.0 ± 8.1
	1/10,000	62.2	307.1 ± 23.2	58.3 ± 7.7

* Indicates a significant difference compared with the control without ester (at least at $P < 0.05$).

concentration on the weight of the larvae ($F = 0.92$, $P < 0.4$). Methyl palmitate at the concentration of 10^{-2} (wt:wt) gave the heaviest larvae, and, in this case, the acceptance and the amount of royal jelly were not different from the controls (Table 1). The weight of the larvae was higher (69.1 ± 1.8 mg) on the bar at the bottom of the frame than on the bar at the top (56.3 ± 2 mg) ($F = 25.32$, $P < 0.0001$).

Table 2. Effects of the esters included in the wax of artificial queen cells on the rearing behavior of the workers, three concentrations pooled

Ester	Worker acceptance of cells (%)	RJ/accepted cell (mg) (mean ± SEM)	Wt of larvae (mg) (mean ± SEM)
10 Esters	67.7	299.8 ± 11.5	62.4 ± 4.4
Ethyl linoleate	61.4	313.2 ± 13.5	63.7 ± 4.7
Ethyl linolenate	69.1	319.3 ± 12.0	65.4 ± 3.9
Ethyl oleate	68.1	315.7 ± 11.7	58.0 ± 3.9
Ethyl palmitate	62.2	308.4 ± 12.4	57.2 ± 3.9
Ethyl stearate	64.2	313.7 ± 11.1	60.2 ± 3.8
Methyl linoleate	58.8	337.3 ± 12.8*	62.5 ± 4.3
Methyl linolenate	60.7	307.9 ± 12.3	67.4 ± 4.8
Methyl oleate	67.6	316.4 ± 11.6	56.8 ± 3.7
Methyl palmitate	69.9	312.6 ± 11.2	77.5 ± 4.8*
Methyl stearate	72.3*	309.3 ± 12.1	57.0 ± 4.0
Control	63.3	306.6 ± 19.9	61.1 ± 6.6

* Indicates a significant difference compared with the control without ester (at least $P < 0.05$).

In the second experiment, based on their efficiency to modulate the acceptance of the cell, the quantity of royal jelly deposited in the cups, and the weight of the larvae (Table 2), the three active compounds, methyl palmitate, methyl linoleate, and methyl stearate, were individually tested at the concentration of 10^{-3} (wt:wt) and compared with the controls. For each compound tested, there was an important effect of the rearing colony ($P < 0.001$) on the production of royal jelly and on the larval weight in the experiments using the different compounds. The acceptance of the wax cups was higher with the three different esters (Fig. 1), but it was not statistically different from the controls. The amount of royal jelly per accepted cup was more important in the cup containing methyl linoleate 10^{-3} (wt:wt) ($F = 7.14$, $P < 0.008$), and also methyl stearate 10^{-3} (wt:wt) ($F = 22.5$, $P < 0.0001$) (Fig. 1). The larval weight was also higher in the cup containing one of the three esters ($F =$

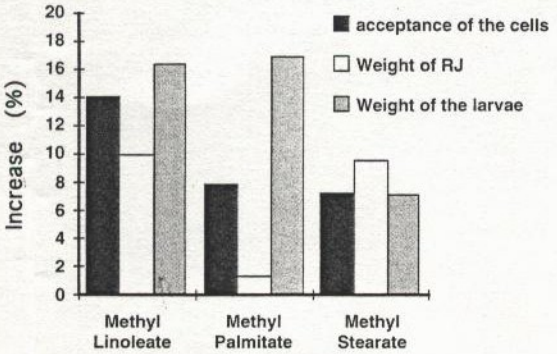

Fig. 1. Acceptance of the cells, royal jelly produced by accepted cell, and weight of the larvae, using one of the three esters, compared with the control without ester.

4.1, $P < 0.05$), but only cells with methyl palmitate 10^{-3} (wt:wt) showed significant differences from the controls (Fig. 1).

The third experiment confirmed the effect of methyl stearate on the acceptance of the cell, of methyl linoleate on the quantity of royal jelly deposited in the cups and of methyl palmitate on the larval weight (Fig. 2 A–C). Active concentrations of methyl stearate and methyl linoleate varied between 10^{-4} and 10^{-3} (wt:wt) of ester in the wax. For methyl palmitate, active concentrations were 10^{-3} and 10^{-1} (wt:wt).

Discussion

The three experiments showed evidence that at least three compounds act as cues to modulate the larval rearing behavior of the worker bees. Methyl stearate produced the best acceptance of the wax cups, methyl linoleate resulted in an increased amount of royal jelly in the accepted wax cups, and methyl palmitate resulted in a production of heavier larvae, which lead us to assume that qualitatively a different royal jelly may be given to the heavier larvae. Thus, methyl linoleate and methyl palmitate appear to induced differential royal jelly production in quantity and quality, respectively.

Behaviorally active concentrations varied between 10^{-4} and 10^{-3} (wt:wt) of ester in the wax using our experimental conditions. Methyl palmitate was also active at the concentration of 10^{-1} (wt:wt), but this concentration corresponded to a larger amount in comparison to the amount naturally found on larvae. A higher amount of methyl linoleate or methyl stearate did not produced increased response. Thus, as we have already shown for the recognition of young and old larvae (Le Conte et al. 1994), the compounds act in a particular range of concentrations.

These esters are of low volatility; using headspace procedure to trap volatile compounds during 24 h, with a threshold of 5 ng, Rickli et al. (1992) did not find any methyl palmitate, ethyl palmitate, or methyl linolenate on 8-d-old larvae, whereas these

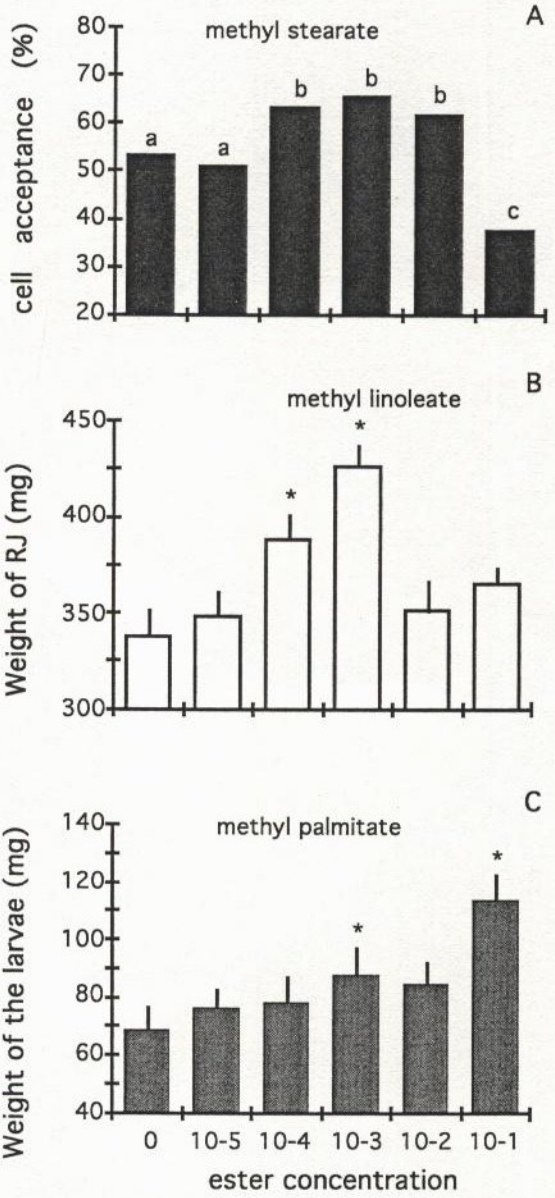

Fig. 2. Effect of ester concentration on the rearing behavior of the workers.

compounds were found in amounts of 55 ng or higher on the worker larval cuticle (Trouiller et al. 1991). In the case of methyl oleate, one of the most volatile esters, <1% of the pure compound was released per 24 h in experimental conditions of headspace at 34°C (Le Conte et al. 1995). Under experimental conditions in our study, each compound was included in an 0.1-g cup of wax. A wax cup containing an ester concentration of 10^{-3} or 10^{-4} (wt:wt) included 10^2 or 10 μg of ester, respectively. Although <1% is released within 24 h, <45, 7, and 15 queen larval equivalent were released per day by the wax cup with methyl pal-

mitate 10^{-3} (wt:wt), methyl stearate 10^{-4} (wt:wt), and methyl linoleate 10^{-4} wt:wt active ester concentrations, respectively. Thus, modulations of the rearing behaviors of the worker bees were obtained by using a small amount of the esters. Previous studies showed that workers respond to lower concentrations of these compounds included in paraffin lures by capping cells or building queen cells (Le Conte et al. 1990, 1994).

Because these products are naturally present on the cuticle of the larvae, we assume that they are involved, at least partly, in the chemical ecology of the feeding behavior of the larvae. Results obtained from these experiments, where esters were used as complementary stimuli to the real larvae, lead us to conclude that, at least three different chemical signals could be perceived by the workers during the process of feeding honey bee larvae.

One ester, methyl stearate, which increased the acceptance of the wax cups, was not found on young larval cuticle (2 to 3 d old), whereas it is a major component on older (8–9 d) larvae (Le Conte et al. 1994). Thus, methyl stearate could be part of the general signal produced by older larvae to indicate their presence to the worker bees, described by Huang and Otis (1991a). Amounts of this compound increase with larval age; that increase could also signal relative investment by workers, thus enhancing larval protection.

Another ester, methyl linoleate, increased the amount of royal jelly deposited by workers in the cells. This compound was found to be one of the major compounds on young larval cuticle; it could correspond to the stimulus linked to the quantity of food present in the cell, which is perceived by the nurses, as part of the quantitative feeding signal reported by Huang and Otis (1991b). It could stimulate nurses to feed a larger quantity of food to larvae possessing higher concentrations of methyl linoleate. Further comparative chemical studies using starved and control larvae should confirm the role of this compound in the feeding process.

The ester, methyl palmitate, generated heavier larvae. Young queen larvae may have 27 fold as much of this compound as young worker larvae (Trouiller 1993), and its presence could correspond to a specific qualitative feeding signal for queen larvae. Further biochemical experiments are needed to explore the difference in composition of the royal jelly obtained with the controls or with methyl palmitate.

The blend of esters, corresponding to 8.5-d-old larvae, did not modulate feeding behavior. This is not surprising because this blend was found to be specific to older (8.5-d-old) larvae and to trigger capping of the cells containing 8.5-d-old larvae (Le Conte et al. 1990). We have found that workers are able to recognize young and old larvae because they produce different blends of these esters which define the chemical signature of the larvae (Le Conte et al. 1994). Although the blend of esters trigger specific behaviors, the esters, tested separately, can induce different responses from the workers, depending on the state of the larvae (old or young larvae, queenless, or queen right colonies). For example, we have shown that methyl palmitate can trigger different behaviors such as the capping of cells, the building of a queen cell, or the increase of the larval weight. We have also shown that worker bees deposited more royal jelly in the wax cups with methyl linoleate 10^{-3} (wt:wt), whereas methyl linoleate is also involved in recognition of young larvae (Le Conte et al. 1994). Thus, the ecological state of the colony, as well as the state of the larvae, can trigger differential worker response toward the compounds.

On a practical level, information could be used from this study for beekeeping purposes.

The effect of the colony appears to be very important in the production of royal jelly and in the weight of the larvae. It is a well-known phenomenon that the rearing of queens requires strong rearing colonies. An interesting point is that some colonies did not decrease the number of larvae they rear to fit the feeding of the larvae they kept. It would be interesting to study the quality of the queens they produce. More experiments are needed to explain the basis of this colony level variation, but we already know that there is an important variability in queen rearing depending on the race of the colony.

The amount of royal jelly and the weight of larvae increased when larvae are reared on the bar at the bottom of the frame rather than on the bar at the top. The position of the bottom bar corresponds to the most common position of the brood area within a frame, whereas the top bar corresponds to the top of the brood and the honey area. The position of the honey bee cluster in a colony is certainly related to these differences. Similar observations were made on the development duration of the queen pupae depending on the place on the bars (DeGrandi-Hoffmann et al. 1993).

The use of the three different active compounds always improved the three factors studied, in comparison to controls. Considering the amount of royal jelly produced by grafted cups; i.e. the amount of royal jelly in the accepted cups per percentage of acceptance, greater amounts of royal jelly were obtained in the first experiment with some types of wax cups than in the control, and among the different esters, methyl stearate produced the most. In the second experiment, the increased amount of royal jelly produced per grafted cup, obtained with the three different compounds separately, varied from +24% in the case of methyl linoleate at 10^{-3} (wt:wt) to +9.5 % for methyl palmitate at 10^{-3} (wt:wt), when compared with the controls, and are related to a best acceptance and an increase of the amount of royal jelly per accepted cell.

The weight of the larvae can be increased depending on the colony and on the place of the bar and also when using methyl palmitate. But further

studies are needed to know the quality of queens obtained by varying these three different factors. Moreover, we can assume that, in the three different experiments, the compounds included in the wax could attract more nurses from the queenright to the queenless part of the colony. In such a case, the controls could also be profiting from the global attraction of the compounds and, thus, our results could be underevaluated.

Because these compounds can lead to an augmentation of the production of royal jelly and can modulate the weight of the larvae, it could be interesting to use them in beekeeping to increase the production of royal jelly and optimize the quality of the young queens in artificial queen rearing. Since we obtained significant results with some combinations and some blends of esters, a patent has been obtained on the use of these substances in the production of royal jelly and in the rearing of queens (Le Conte et al. 1993).

These studies, coupled with the work of earlier research (Le Conte et al. 1990, 1994, 1995) demonstrate the behavioral effect of a brood pheromone made of these esters. The pheromonal blend can also act, in experimental conditions, on the inhibition of the queenless worker ovaries (Le Conte 1990, Arnold et al. 1994) and on the development of the hypopharyngeal glands (Le Conte 1990). Moreover, methyl palmitate (Rickli et al. 1992), ethyl palmitate and methyl linolenate, are used by the mite, *Varroa jacobsoni,* as a chemical cue to find its host (Le Conte et al. 1989). Thus, these chemical compounds should be considered as major components in the chemical ecology of the honey bee colony.

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